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RESEARCH ARTICLE

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Evaluation of seasonal changes of triterpenic acid contents in *Viscum album* from different host trees

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ABSTRACT

Context: *Viscum album* L. (Loranthaceae) is a semi-parasitic plant used in pharmacy and medicine mostly for its hypotensive and anticancer activity. The effects may be related to the presence of triterpenic acids, such as betulinic (BA) and oleanolic (OA) acids.

Objectives: In our investigations the content of triterpenic acids in *V. album* from different host trees depending on the season of harvest was determined.

Material and methods: *V. album* herb was dried and extracted with ethyl acetate using ultrasound energy. The reversed phase HPLC-PDA method was used for the analysis of triterpenic acids. The structure of the target components was confirmed by mass spectrometry with an electrospray ionization source.

Results: Diversity in the content of both compounds was noted; however, OA was the dominant triterpenic acid and the amount thereof was ~10 times higher than that of BA. The analysis of changes in the amount of triterpenic acids during the spring-winter period revealed the highest content of OA in summer (from 6.84 to 13.65 mg/g). In turn, in the other seasons of harvest, the content was in the range of 4.41–9.83, 6.41–9.56 and 5.59–12.16 mg/g for spring, autumn and winter, respectively. In most cases, a similar tendency was observed for BA.

Discussion and conclusion: In most cases, the highest amount of the investigated compounds was found in summer; thus, this period seems to be optimal for acquisition of plant material rich in triterpenic acids.

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Introduction

Viscum album L. (Loranthaceae) (mistletoe) is a perennial, evergreen and semi-parasitic plant. Since ancient times, it has been used in European and Asian folk medicine for treatment of many diseases such as epilepsy, diabetes mellitus, cancer, hypertension, headache and rheumatoid arthritis. It contains various biologically active constituents such as lectins, viscotoxins (Eremia et al. 2008) flavonoids (Lyu et al. 2000; Orhan et al. 2002), polysaccharides, alkaloids, terpenoids (Orhan et al. 2001), saponins, tannins, phytosterols and phenolic acids (Łuczkiewicz et al. 2001; Vicaş et al. 2011). The composition and quantity of constituents may vary significantly because *V. album* is able to infect numerous tree species and the host is an important factor for its phytochemical profile and bioactivity (Vicaş et al. 2011; Orhan et al. 2014; Orhue et al. 2014). Therefore, the plants from different trees are traditionally used for various purposes, e.g., mistletoe growing on guava, kolanuts, and citrus is effective in treatment of cancer, hypertension and nervousness (Ekhaise et al. 2010), that from pear (*Pyrus* L.) is employed as a cardiovascular drug, whereas mistletoe from hawthorn (*Crataegus* L.) exhibits mainly hypotensive action (Panossian et al. 1998).

Nowadays, a number of *in vivo* and *in vitro* studies confirm the broad spectrum of the therapeutic action of *V. album*. There are many reports about its anti-inflammatory (Hegde et al. 2011), antinociceptive, hypotensive (Ofem et al. 2007), antidiabetic (Orhan et al. 2005), anticancer (Burger et al. 2001), immunomodulatory (Lavastre et al. 2004), antioxidant (Orhan et al. 2005), antimicrobial (Orhue et al. 2014), antiepileptic, sedative, antipsychotic (Gupta et al. 2012) and cytotoxic activities (Cebović et al. 2008). *V. album* is used in contemporary pharmacy and medicine in a form of commercially available extracts and preparations mostly for treatment of hypertension and some cases of cancer.

Some pharmacological effects may be related to the presence of triterpenic acids, such as oleanolic and betulinic acid, e.g., the extract from *V. album* containing triterpenic acids is effective in acute lymphoblastic and myeloid leukemia (Delebinski et al. 2012, 2015) and induces apoptosis of murine melanoma cells (Strüh et al. 2012). Moreover, numerous investigations have demonstrated that oleanolic acid (OA) decreased blood pressure, which is attributed to the diuretic and antioxidant action (Somova et al. 2003; Bachhav et al. 2011) and exerted cytotoxic (Li et al. 2002, 2013; Kartini et al. 2014), anti-inflammatory, and hepato- and nephroprotective effects (Liu 2005; Patil et al. 2010). Betulinic acid (BA) also shows cytotoxic and antitumor activity (Srivastava et al. 2010).

There are some publications regarding triterpenic acids in *V. album* (Jäger et al. 2007, 2009) and only one report describes a comparison of their content in mistletoe from different hosts (Kyung et al. 2013). However, the production of biologically active substances in plants is strongly related with the vegetation period (Barbasz et al. 2012); thus, the season of harvest is one of the key parameters to obtain the plant material with high

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Table 1. Host of *V. album* L. ssp. *album*, sites and localities.

Sample no.	Host	Family	Site	Coordinates
1.	*Robinia pseudoacacia* L.	Leguminosae	Tychowo (forest)	53°90N, 16°28E
2.	*Sorbus aucuparia* L.	Rosaceae	Tychowo (roadsite)	53°93N, 16°26E
3.	*Betula pendula* Roth	Betulaceae	Tychowo (forest)	53°91N, 16°29E
4.	*Tilia cordata* Mill.	Malvaceae	Buchałowice (garden)	51°34N, 22°17E
5.	*Malus* 'Jonathan'	Rosaceae	Lublin (Botanical garden)	51°26N, 22°51E
6.	*Populus nigra* L.	Salicaceae	Krosinko (forest)	53°91N, 16°27E
7.	*Pinus sylvestris* L.	Pinaceae	Górna Owczarnia (forest)	51°11N, 21°98E

amounts of valuable components. Therefore, the aim of our work was to evaluate the OA and BA content in mistletoe in different seasons. To the best of our knowledge, the study on seasonal changes in their concentration was carried out for the first time. Our results may be useful to obtain plant material rich in triterpenic acids.

Materials and methods

Chemicals

Triterpenic acid standards were purchased from Sigma (St. Louis, MO). The purities of the standards were ≥98% and ≥97% for betulinic (BA), and oleanolic (OA) acid, respectively. Ethyl acetate, methanol and HPLC-grade acetonitrile were purchased from Merck (Darmstadt, Germany). Water was deionized and purified by ULTRAPURE Milipore Direct-Q® 3UV–R (Merck, Darmstadt, Germany).

Plant material

V. album L. ssp. *album* was collected from different hosts in Poland in 2013 (October) and in 2014 (January, April and July), and identified in the Department of Pharmaceutical Botany Medical University of Lublin by Prof. A. Bogucka-Kocka. Voucher specimen numbers are deposited in the Botanical Garden of UMCS. Host plants and localities are presented in Table 1.

Sample preparation

V. album was dried in 40 °C, pulverized and accurately weighted (~1 g). Samples were extracted with a 25 mL portion of ethyl acetate in an ultrasonic bath at a temperature of 35 °C during 30 min. The procedure was repeated three times with a fresh portion of the solvent. The combined extracts were concentrated in a rotary vacuum evaporator to 10 mL.

Quantification

Quantitative HPLC analysis was conducted using a VWR Hitachi Chromaster 600 chromatograph (Merck, Darmstadt, Germany) with a pump (5160), a degasser, thermostat (5310), autosampler (5260), PDA detector (5430) and EZChrom Elite software.

The extracts were separated on a LiChrospher 100 (Merck, Darmstadt, Germany) C18 reversed-phase column (25 cm ×4.0 mm i.d., 5 µm particle size) at a flow rate of 1.0 mL/min with the use of isocratic elution. The mobile phase consisted of acetonitrile:water:1% phosphoric acid (80:20:0.5 v/v/v). The temperature of the autosampler and column thermostat was 10 °C. The data were collected in the wavelength range from 200 to 400 nm. The quantification was conducted at 200 nm. The chromatographic fractions eluted at retention time characteristic for OA and BA were collected using a Foxy R1 fraction collector (Teledyne Isco, Lincoln, NE) and investigated by a direct injection on mass spectrometer micrOTOF-Q II (Bruker Daltonics, Bremen, Germany) with electrospray ionization (ESI). Mass spectrometric data were analyzed with the use of Compass DataAnalysis software version 4.1 (Bruker Daltonics, Bremen, Germany).

Statistical analysis

The STATISTICA ver.10 (StatSoft Inc., Tulsa, OK) program was used for statistical evaluation. The data were analyzed by ANOVA, the significances of differences were examined using Fisher's LSD test. The confidence level was set at $p = .05$.

Results and discussion

Extraction and HPLC conditions

The solvent, extraction method and chromatographic conditions were chosen on the basis of our earlier investigation (Wójciak-Kosior & Sowa 2009; Wójciak-Kosior et al. 2013). The mobile phase composition was slightly modified to separate oleanolic and betulinic acid from the interfering constituents of the plant extract. The reduction of the analysis temperature to 10 °C improved significantly the resolution of both acids (Wójciak-Kosior & Sowa 2009).

Examples of chromatograms are presented in Figure 1. The identity of the compounds was established by comparison of retention times and spectra with corresponding standards. The purity of the chromatographic peaks obtained was checked by acquisition of spectra at three different peak sections: upslope, apex, and downslope and comparison with the reference spectrum. The similarity factor calculated by the EZChrom Elite software was higher than 0.98. Moreover, the chromatographic fractions eluted at retention time characteristic for OA and BA were (Merck, Darmstadt, Germany) collected and the structures were confirmed by direct injection mass spectrometry.

Method validation

The method was validated for linearity, precision, and accuracy. Calibration plots were established by analysis of standard solutions at seven different concentration levels. The mean peak areas ($n = 5$) were taken for the construction of the calibration curve. The data were analyzed by a linear regression least square model. The accuracy of the method was established by performing recovery experiments at three different levels. About 50, 100 and 150% of the determined amount of BA and OA were added to the plant extract and the recovery was calculated on the basis of differences between the amount added and quantified.

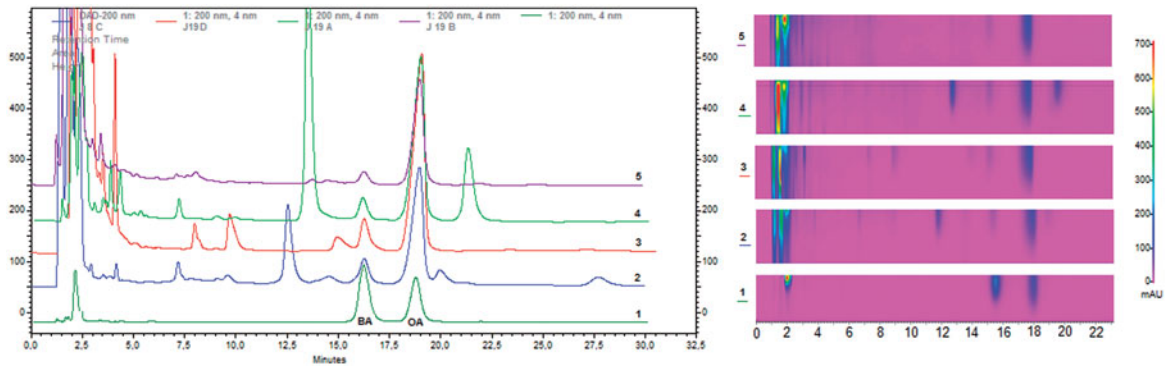

Figure 1. 2D and 3D chromatograms of extracts from *V. album* (host *Malus* 'Jonathan') harvested in different seasons. Line 1-standards of oleanolic (OA) and betulinic acid (BA); line 2-spring; line 3-summer; line 4-autumn; line 5-winter.

Table 2. Validation data obtained for quantification of triterpenic acids.

Compound	Concentration range (mg/mL)	Correlation coefficient (r)	Regression equation	Precision (% RSD)	Recovery (%)
BA	0.01–0.5	.9998	y = 62898x + 17711	0.41–1.11	97.3–98.9
OA	0.2–1.5	.9999	y = 55925x + 126773	0.37–0.95	98.3–99.4

Table 3. Changes in the oleanolic acid content in *V. album* from different host depending on seasons (mg/g ± SD).

Host	Spring	Summer	Autumn	Winter
R. pseudoacacia L.	4.411 ± 0.254^{k}	$9.017 \pm 0.549^{h,i,j}$	$7.794 \pm 0.311^{e,f,g}$	$7.091 \pm 0.418^{c,d}$
S. aucuparia L.	$6.216 \pm 0.362^{a,b}$	$8.106 \pm 0.484^{f,g}$	$7.134 \pm 0.344^{c,d}$	$7.211 \pm 0.427^{c,d,e}$
B. pendula Roth	5.281 ± 0.101^{l}	$6.836 \pm 0.402^{b,c,d}$	$6.412 \pm 0.415^{a,b,c}$	$6.776 \pm 0.392^{b,c,d}$
T. cordata Mill.	$6.954 \pm 0.401^{b,c,d}$	$8.509 \pm 0.505^{g,h,i}$	$7.175 \pm 0.550^{c,d,e}$	$8.615 \pm 0.518^{g,h,i}$
M. 'Jonathan'	5.799 ± 0.342^{a}	10.758 ± 0.614^{j}	9.562 ± 0.408^{j}	5.591 ± 0.320^{a}
P. nigra L.	6.049 ± 0.329^{a}	13.654 ± 0.808^{m}	$6.731 \pm 0.380^{b,c,d,f}$	$7.480 \pm 0.449^{d,e,f}$
P. sylvestris L.	$9.83 \pm 0.541^{j,h}$	$8.205 \pm 0.471^{f,g,h}$	$9.318 \pm 0.469^{i,j}$	12.161 ± 0.612^{n}

The significances of differences were examined using Fisher's test ($p < .05$). The data followed by the same letters are not significantly different.

Table 4. Changes in the betulinic acid content in *V. album* from different host depending on seasons (mg/g ± SD).

Host	Spring	Summer	Autumn	Winter
R. pseudoacacia L.	$0.384 \pm 0.048^{a,b}$	$0.896 \pm 0.111^{f,g,h}$	$1.098 \pm 0.126^{h,i,j}$	$0.404 \pm 0.052^{a,b,c}$
S. aucuparia L.	0.362 ± 0.049^{a}	$0.914 \pm 0.101^{f,g,h}$	$0.494 \pm 0.064^{b,c,d}$	$0.922 \pm 0.131^{f,g,h,i}$
B. pendula Roth	$0.750 \pm 0.098^{e,f}$	$1.137 \pm 0.141^{h,i,j,k}$	$0.982 \pm 0.122^{g,h,i}$	$0.905 \pm 0.124^{f,g,h,i}$
T. cordata Mill.	$1.185 \pm 0.138^{i,j,k}$	$1.370 \pm 0.160^{k,l}$	1.567 ± 0.181^{l}	$1.098 \pm 0.121^{h,i,j}$
M. 'Jonathan'	$0.825 \pm 0.102^{e,f,g}$	$1.302 \pm 0.166^{j,k,l}$	$0.773 \pm 0.092^{e,f}$	$0.431 \pm 0.052^{a,b,c}$
P. nigra L.	0.374 ± 0.046^{a}	$1.405 \pm 0.179^{k,l}$	0.555 ± 0.070^{d}	$0.509 \pm 0.063^{c,d}$
P. sylvestris L.	2.111 ± 0.270^{m}	0.676 ± 0.062^{e}	$0.929 \pm 0.105^{f,g}$	2.125 ± 0.26^{m}

The significances of differences were examined using Fisher's test ($p < .05$). The data followed by the same letters are not significantly different.

The validation parameters obtained such as high linearity (r > .9998), precision (relative standard deviation from 0.37 to 1.11% for standard solutions), and accuracy (recovery above 97.3%) were satisfactory for quantitative analysis. The data are summarized in Table 2.

Content of triterpenic acids in plant material

Mistletoe is a semi parasitic plant and its metabolite content is associated with the vegetation period of the host; thus, the quantity of components may vary depending on the season of harvest, e.g., Barbasz et al. (2012) observed significant changes in the content of sugars, lipids and polyamines in different periods of the year.

Since the recent reports show high activity of extracts containing triterpenes or triterpenic acids isolated from mistletoe, in the present research, the content of oleanolic and betulinic acid in *V. album* was investigated depending on the season. The plant material was collected from the same trees in spring (April), summer (July), autumn (October) and winter (January) and next dried, extracted and target compounds were determined by the HPLC-PDA method. The average values ($n = 3$) of the OA and BA contents determined in 1 g of dried plants are presented in Tables 3 and 4, respectively.

As shown in our research, OA was the dominant triterpenic acid. Its amount was several times higher than that of BA and it was in the range from 4.41 to 13.65 mg/g of dry weight. The highest content of OA, above 12 mg/g was observed in summer for *V. album* from *Populus nigra* L. (Salicaceae) and in winter from *Pinus sylvestris* L. (Pinaceae). In turn, the lowest amount (below 5 mg/g) was found in mistletoe from *Robinia pseudoacacia* L. (Leguminosae) in spring. The average concentration of OA was 7.81 mg/g. The results obtained are slightly higher than values reported for the Korean mistletoe *(V. album* var. *coloratum)* (Kyung et al. 2013). The BA concentration ranged from 0.36 to 2.41 mg/g of dry weight and the highest amount (above 2 mg/g) was found in mistletoe from *P. sylvestris* in spring and winter. The average BA content was 0.96 mg/g.

As can be seen in Tables 3 and 4, a significant fluctuation of the content of both acids was observed depending on the period of the year; however, the tendency for *V. album* derived from Angiospermae (host number 1–6) was different than in that collected from *P. sylvestris* (Gymnospermae), host number 7. Generally, for samples 1–6, the highest content of the investigated compounds was found in summer (from 6.84 to 13.65 mg/g for OA and from 0.90 to 1.41 mg/g for BA). In turn, in the other harvest seasons, the content ranges were as follows, for OA: 4.41–6.95, 6.41–9.56 and 5.60–8.61 mg/g and for BA: 0.36–1.18, 0.49–1.57 and 0.40–1.10 mg/g for spring, autumn and winter, respectively. The average content of OA was 5.78, 9.48, 7.47 and 7.13 mg/g, while for BA it amounted to 0.65, 1.17, 0.91 and 0.71 mg/g for spring, summer, autumn and winter, respectively. A different relationship between the period of harvest and the content of the investigated triterpenic acids was observed for *V. album* from *P. sylvestris*. The highest level of both compounds was detected in winter (12.16 mg/g of OA and 2.13 mg/g of BA), while in summer it decreased significantly by ∼33 and 68% for OA and BA, respectively.

Conclusion

In our research, the content of triterpenic acids in *V. album* from different hosts and in various seasons was determined. A high variance of the OA and BA content was noted; however, OA was a dominant triterpenic acid, and the quantity thereof was ∼10 times higher than that of BA. In most cases, the highest amount of the investigated compounds was found in summer; thus, this period seems to be optimal for acquisition of plant material rich in triterpenic acids.

Disclosure statement

The authors report no declarations of interest.

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